FEBS 28860 FEBS Letters 576 (2004) 423–427

Transcriptomic responses to cadmium in the ectomycorrhizal fungus *Paxillus involutus*

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Received 12 August 2004; revised 14 September 2004; accepted 14 September 2004

Available online 25 September 2004

Edited by Ned Mantei

Abstract The molecular mechanisms underlying the response of ectomycorrhizal fungi to heavy metals in general and cadmium in particular remain poorly understood. We screened 2040 arrayed cDNAs of the ectomycorrhizal fungus *Paxillus involutus* to identify cadmium-responsive genes by using differential hybridization. Forty nine (2.4%) of the 2040 cDNAs were differentially expressed, among which transcripts coding a laccase, an aconitase, and a metallothionein were upregulated by 3.9-, 3.7- and 2.8-fold, respectively, whereas genes coding hydrophobins and threonine dehydratase were strongly downregulated. Our results suggest that complexation of cadmium by phenolic compounds, or by complexing peptides such as metallothioneins, is probably key determinant of the cellular response to cadmium in *P. involutus*. In addition, the present study suggests that the synthesis of hydrophobins may be efficiently reduced, thus redirecting Cys to the manufacture of Cys-enriched compounds.

Keywords: cDNA array; Cadmium responding gene; Ectomycorrhizal fungus; *Paxillus involutus*

1. Introduction

Cellular mechanisms involved in metal detoxification and tolerance have been reviewed and the role of ectomycorrhizal fungi in ameliorating the effects of metal toxicity on the host plant emphasized [1,2]. Mycorrhizal fungi can occupy industrially degraded habitats, which are often contaminated by heavy metals such as lead, zinc, cadmium, copper, and nickel [3]. Although interactions of metals with fungi in general [2] and with mycorrhizal fungi in particular [4,5] have been reviewed, little attention has been paid to the molecular responses of ectomycorrhizal fungi towards cadmium. We have also hypothesized the fact that mycorrhizal birch could better meet their N demand under metal stress, compared to non-mycorrhizal plants [6]. An altered growth of mycorrhizal species was found when metals were added to the culture medium, but considerable variations could be observed depending on fungal species [7,8] or isolates [9]. Resistance to heavy metals can be achieved either by avoidance (restriction of metal uptake or increased efflux, extracellular formation of complexes) or by tolerance (intracellular chelation of metals, compartmentation in vacuoles) [5]. Metal uptake and compartmentation mechanisms in the ectomycorrhizal fungus *Paxillus involutus* were examined, and it was demonstrated that cadmium is partly retained by cell wall components and partly actively taken up by fungal cells and further distributed between cytosolic and vacuolar compartments [10]. Metal stress may also induce other cellular changes in *P. involutus* such as activation of a manganese-superoxide dismutase at the post-transcriptional level [11]. In the present study, we have investigated the molecular determinants of metal response in the ectomycorrhizal fungus *P. involutus*, using a cDNA array analysis to identify differentially expressed genes.

2. Materials and methods

2.1. Growth conditions and cadmium treatment

The ectomycorrhizal fungus used was an isolate of *P. involutus* (Batsch) Fr. (ATCC 200175). It was grown on cellophane covered agar medium containing modified Melin-Norkrans medium (MMN) [8]. Exponentially growing (7-day-old) colonies were transferred onto liquid modified MMN medium (pH 4.5) for an adaptation period of 3 days, with a daily change of the medium. After this period, $CdSO_4$ was added at a final concentration of 0.05 or 5 ppm of Cd^{2+} under sterile conditions for various time periods.

2.2. RNA isolation and cDNA library construction

Total RNA isolation from *P. involutus* grown with or without cadmium was performed as described previously [11]. Double-stranded cDNAs were synthesized from mixed RNA populations as starting material using the Smart™ PCR cDNA library construction kit (Clontech Laboratories Inc., Palo Alto, CA). PCR products were directly ligated into the plasmid vector pGEM-T (Promega, Madison, WI) and transferred into DH5αF′ competent cells. Bacterial clones were stored at −70 °C in 35% glycerol.

2.3. Generation and analysis of cDNA arrays

cDNA inserts of bacterial clones were amplified by PCR with the primers SP6 and T7 using the following parameters: 92 °C for 2 min; 35 cycles at (92 °C for 1 min; 56 °C for 45 s; and 72 °C for 2 min); and a final extension at 72 °C for 10 min, in 50 μl reaction volumes. Successful production of PCR products was confirmed by agarose gel electrophoresis. To investigate changes in gene expression profiles when the ectomycorrhizal fungus *P. involutus* was grown in the presence of cadmium, cDNA arrays were assembled with 2040 cDNAs. The 2040 PCR-amplified cDNAs inserts (about 20–30 ng/μl) were spotted in duplicate onto six nylon membranes by using the *Bio*Grid arrayer (*Bio*Robotics, Cambridge, UK) according to the

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.09.028

manufacturer's instructions (Eurogentec, Seraing, Belgium). Complex cDNA probes were amplified using total RNA from control and cadmium-stressed fungal colonies, then labeled and hybridized to nylon microarrays as described previously [12,13]. Three biological replicates were performed for the two conditions studied. Images of hybridized membranes obtained with a Phosphoimager (Bio-Rad Laboratories, Hercules, CA) were analyzed using Xdotreader (Cose, Paris, France) to obtain raw spot intensity data. Local background value for each membrane was calculated on the basis of areas with no DNA spotted. After background subtraction, only values greater than 2-fold above the background value were further normalized and analyzed. To this end we used (i) the robust scatter plot smoother 'lowess' from the GeneSpring™ software version 5.1 (Silicon Genetics), which performs locally linear fits, to execute an intensity-dependent normalization of the log ratios [14] and (ii) the CyberT statistics program (version available at http://visitor.ics.uci.edu/genex/cybert/). In CyberT, we used a Bayesian statistical method, based on the *t* test, conveniently used to test for statistically significant differences between gene expression in the two conditions tested [15]. The GeneAnova software [16] was used to check the correlation between the repetitions of each condition: Cd-treated and control mycelia.

2.4. Sequencing and cDNA sequence analysis

Cadmium-responsive clones, according to the array hybridizations, were sequenced. DNA sequencing was performed as described [11]. Homology searches in databases were performed by using the BLAST program [17] of the National Center for Biotechnology Information and the BEAUTY post-processing program provided by the Baylor College of Medicine [18,19]. The EST sequences have been deposited in the GenBank dbEST at the National Center for Biotechnology Information (NCBI) and their accession numbers are given in Table 1.

2.5. Gene expression analysis

For reverse transcriptase-mediated PCR analysis, DNase-treated RNAs were converted to cDNAs using the Omniscript reverse transcriptase according to the manufacturer's protocol (Qiagen, Hilden, Germany). Reverse transcription (RT) reactions were performed for 60 min at 37 °C and RT products were used in PCR. The reverse (RP) and forward (FP) primers used for RT-PCR analysis for genes coding an aconitase (*ACO*), a hydrophobin (*HYD*), a laccase (*LAC*), and a concanamycin-induced protein type C (*CIPC*) are the following: *ACO* (268 bp), RP 5′-CCCACGGCCTATGAAGGTCA, FP 5′-TGGGAGAAGCGACAAAAGCA; *HYD* (229 bp), RP 5′-GGGCAGGCCAACTACCCACT, FP 5′-CCGAGGTACTCGGCCAACAA; *LAC* (306 bp), RP 5-TCTGGTTGAATCGGGCACTG, FP 5′-ATAGGTCCACGGGCCAGCTC; *CIPC* (373 bp), RP 5′-GGGGGATCCTCAGTAGCGATCTTT, FP 5′-GGGGAAGCTTATGCCCCACCACGAT. The products were amplified by PCR using the following conditions; 94 °C for 3 min followed by 29, 26, 28 and 27 cycles for *ACO*, *HYD*, *LAC* and *CIPC*, respectively, at 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min using an Eppendorf Mastercycler (Eppendorf, Le Pecq, France). The suitability of the extracted RNA for RT-PCR amplification was checked by performing RT-PCR control experiments with *CIPC*. This control gene was chosen from those showing no differential expression on cDNA arrays. RT-PCR products were run on a 2% agarose gel.

Table 1
Differential gene expression in cadmium-treated versus non-treated *P. involutus* mycelium

Accession #	Highest homology (organism)[a]	*P* value	Ratio Cd/N[b]	Bayes.lnp[c]	PPDE (p)[d]
CN072106	No match		− 8.9	$3.9\ e^{-15}$	1
CN072107	No match		− 6.6	$1.6\ e^{-13}$	1
CN072109	Hydrophobin-3 precursor (*Pisolithus tinctorius*)	$9.8\ e^{-8}$	− 4.3	$6.1\ e^{-13}$	1
CN072110	Hydrophobin-3 precursor (*P. tinctorius*)	$4.2\ e^{-5}$	− 4.0	$2.4\ e^{-12}$	0.99
CN072113	Threonine dehydratase (*S. typhimurium*)	$1\ e^{-20}$	− 3.4	$7.7\ e^{-11}$	0.99
CN072115	Threonine dehydratase (*Synechocystis sp.*)	$3.1\ e^{-15}$	− 3.0	$1.8\ e^{-9}$	0.99
CN072144	Threonine dehydratase (*Bacillus cereus*)	$3.7\ e^{-31}$	− 3.0	$1.0\ e^{-9}$	0.99
CN072124	Conserved hypothetical protein (*Schizosaccharomyces pombe*)	$2.6\ e^{-23}$	− 2.0	$1.6\ e^{-4}$	0.96
CN072145	Inorganic pyrophosphatase (*Kluyveromyces lactis*)	$1.0\ e^{-90}$	−1.6	$9.9\ e^{-5}$	0.97
CN072151	Inorganic pyrophosphatase (*K. lactis*)	$1.1\ e^{-95}$	−1.6	$1.2\ e^{-4}$	0.97
CN072146	60S ribosomal protein L8 (*S. pombe*)	$7.5\ e^{-33}$	−1.5	$8.7\ e^{-4}$	0.87
CN072141	Probable cystathionine gamma-synthase (*S. pombe*)	$6.2\ e^{-12}$	−1.5	$5.8\ e^{-4}$	0.90
CN072140	Tissue specific transplantation antigen P35B (*Homo sapiens*)	$1.0\ e^{-37}$	−1.5	$3.9\ e^{-4}$	0.93
CN072150	40S ribosomal protein S12 (*Oreochromis niloticus*)	$8.8\ e^{-23}$	−1.5	$7.3\ e^{-4}$	0.88
CN072152	Hypothetical protein (*S. pombe*)	$1.1\ e^{-17}$	1.5	$5.1\ e^{-4}$	0.91
CN072142	Probable malate dehydrogenase, mitochondrial precursor (*Caenorhabditis elegans*)	$2.1\ e^{-122}$	1.5	$8.8\ e^{-4}$	0.87
CN072143	Putative tripeptidyl peptidase I (*Mus musculus*)	$1.8\ e^{-18}$	1.6	$1.4\ e^{-4}$	0.97
CN072166	Translation initiation factor 3 (*S. cerevisiae*)	$5.6\ e^{-28}$	1.7	$4.0\ e^{-5}$	0.99
CN072158	Polyubiquitin 5 (*Phanerochaete chrysosporium*)	$1.9\ e^{-45}$	1.8	$5.1\ e^{-5}$	0.99
CN072149	Proteasome 26S subunit (*H. sapiens*)	$2.3\ e^{-12}$	1.9	$6.7\ e^{-6}$	0.99
CN072153	Putative alanine aminotransferase (*Arabidopsis thaliana*)	$1.3\ e^{-23}$	1.9	$2.9\ e^{-6}$	0.99
CN072180	Heat-shock protein 80 (*Euphorbia esula*)	$6.5\ e^{-27}$	1.9	$3.9\ e^{-6}$	0.99
CN072154	Related to AP-1-like transcription factor (*Neurospora crassa*)	$2.3\ e^{-12}$	2.0	$1.3\ e^{-6}$	0.99
CN072131	Plasma membrane H^+-ATPase 1 (*Filobasidiella neoformans*)	$9.9\ e^{-8}$	2.1	$3.0\ e^{-7}$	0.99
CN072132	Glutamine synthetase (*Agaricus bisporus*)	$5.2\ e^{-58}$	2.3	$1.5\ e^{-8}$	0.99
CN072137	Metallothionein (*A. bisporus*)	2.9 e-06	2.8	$2.3\ e^{-10}$	0.99
CN072147	Aconitase (*C. elegans*)	$1.4\ e^{-22}$	3.7	$2.3\ e^{-11}$	0.99
CN072139	Laccase (*Pleurotus ostreatus*)	$2.3\ e^{-18}$	3.9	$4.4\ e^{-10}$	0.99

Fifty-one genes with the highest and lowest cadmium-/non-treated mycelia expression ratio are listed.
[a] Derived from WU-BLASTX score; *P* value, probability that the observed similarity had occurred by chance.
[b] Ratio of normalized hybridization values of transcripts expressed in the cadmium-treated and in the non-treated mycelium.
[c] *P* value associated with the *t* test on log transformed data using the Bayesian deviation.
[d] Posterior probability of differential gene expression.

2.6. PPO and polyphenol measurements

Mycelia grown on 0, 0.05 or 5 ppm cadmium were ground in 50 mM acetate buffer, pH 5.0, and homogenized for 1 min. Phenol oxidase activities were measured spectrophotometrically using 10 mM DOPA (3,4-dihydroxyphenylalanine) as the substrate, according to [20]. Samples were incubated in the dark at 37 °C for 1 h. Activity was quantified by measuring absorbance at 460 nm against a negative control (omitting DOPA) using a spectrophotometer and expressed as absorption (A) units per gram fresh weight. One A unit $= \Delta A_{460}$ of 1.0/h in the reaction volume. Laccase activity was measured according to a method described elsewhere [21]. The phenolic content of culture medium was measured at 255 nm, according to [22].

3. Results

In this study, *P. involutus* was grown under standard conditions or submitted to an acute cadmium stress (5 ppm for 24 h). To ensure that the acute cadmium stress applied was not lethal for the mycelium, we performed recovery tests, which indicate that mycelium could recover within a few days when transferred to cadmium-free fresh medium (not shown). Moreover, total RNA quantification indicated that the amounts of total RNA did not vary significantly between control and cadmium-treated mycelium for 12 or 24 h. These observations indicate that the cadmium treatment used in our study was not lethal to the mycelium. However, after 48 h of 5 ppm Cd-treatment the amounts of RNA decreased dramatically.

Principal component analysis performed with the GeneAnova software demonstrated that transcript profiles were statistically different between the two conditions analyzed (data not shown). Moreover, there was no significant variation between the three independent replicates (there was no more than 14% variation in signal intensity between the three replicates within one treatment), and redundant clones and duplicates showed similar expression levels on a single membrane. Fold differences in signal intensity between hybridizations with cDNA from control- and Cd-treated mycelia were distributed between (−8.9) and (+3.7). Forty nine (2.4%) of the 2040 cDNAs were differentially expressed with a P value $<1.e^{-3}$ (associated with the t test on log transformed data using the Bayesian deviation). Fifty percent of these differentially expressed transcripts showed no apparent homology to any gene of known function. Thus, the majority of the cadmium-responsive, differentially expressed cDNAs encode novel proteins. Database search results using WU-BLASTX search [18] for the *P. involutus* homologs of known genes are given in Table 1.

A 3.9-fold increase in concentration was found for laccase transcripts, which was confirmed by RT-PCR analysis (Fig. 1). In addition, we measured the activity of total polyphenoloxidase and laccase, and the production of polyphenols, in colony grown for various lengths of time with 0.05 and 5 ppm cadmium. As shown in Table 2, the activity of total polyphenoloxidases, including laccase activity, peaked after 24 h and 4 days of 5 and 0.05 ppm Cd treatment, respectively, followed by an increase of polyphenol production in the liquid medium. After 72 h of 5 ppm Cd treatment, the activity of total polyphenoloxidase and laccase decreased to undetectable levels, which is probably due to the strong decrease in RNA synthesis, as mentioned earlier. After prolonged incubation periods with 5 ppm Cd, these polyphenols accumulated as dark droplets on the top of the colony (not shown).

Cadmium exposure also induced the expression of mRNAs encoding proteins involved in carbohydrate metabolism (α-galactosidase), glycolysis (fructose-bisphosphate aldolase) and citric acid cycle (aconitase) (Table 1). RT-PCR analysis confirmed that Cd induced an upregulation of aconitase in *P. involutus*, a key enzyme of the tricarboxylic acid (TCA) cycle (Fig. 1).

Expression levels of *MT1* transcripts showed a 2.8-fold upregulation in Cd-treated mycelium. A gene coding the yAP-1-like transcription factor was also upregulated under Cd stress, which is in agreement with previous observations in yeast [23].

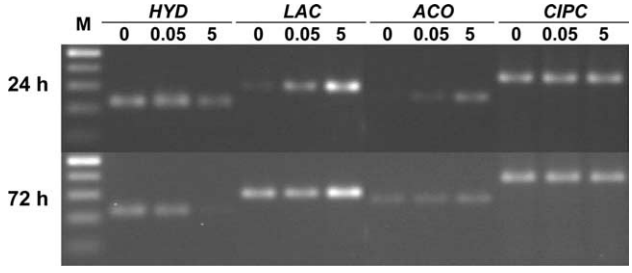

Fig. 1. Transcript levels of *HYD* (hydrophobin), *LAC* (laccase), *ACO* (aconitase) and the control gene *CIPC* (concanamycin-induced protein type C) after RT-PCR using RNA from *P. involutus* cultures supplemented or not with 0.05 or 5 ppm Cd for 24 or 72 h. Lane M: 100 bp DNA Step ladder (Promega). RT-PCR conditions are described in Section 2.

Table 2
Changes in polyphenoloxidase (Ppo) and laccase activities of *P. involutus* mycelial extracts and phenolic content of culture medium during cadmium exposure

Time	[Cd] (ppm)	L-DOPA PPO activity (%)	L-DOPA + 100 μM SHAM laccase activity (%)	Polyphenol OD_{255} (au)
24 h	0.05	109	154	0.12
	5	179	358	0.92
72 h	0.05	144	152	0.16
	5	nd	nd	1.72
4 d	0.05	379	262	0.18
	5	nd	nd	nd
10 d	0.05	nd	nd	0.81
	5	nd	nd	nd

Ppo and laccase activities were quantified in cadmium-treated and control mycelia after various exposure times. Activity values are expressed as % of the control (0 ppm Cd) from the same incubation time. The control values (100%) varied between 25 and 35, and between 3 and 12 μmol/h/mg mycelial fresh weight, for PPO and laccase, respectively. The phenolic content of culture media from cadmium-treated mycelium was measured at 255 nm. The OD_{255} (au, arbitrary unit) for control cultures varied between 0.10 and 0.25. Nd: not determined. L-DOPA: 3,4-dihydroxyphenylalanine. SHAM: salicylhydroxamic acid.

A gene encoding glutamine synthetase (GS) involved in the N metabolism was upregulated 2.3-fold after Cd exposure. The upregulation of this gene upon Cd exposure might be related to a higher demand of nitrogenous compounds, especially Glu, for the synthesis of non-protein thiols such as glutathione (GSH, a Glu–Cys–Gly tripeptide), whose level increased dramatically under Cd stress [24].

A 4.3- and 4.0-fold downregulation was observed for transcripts encoding hydrophobin-3 precursors. Since the EST sequences for these two clones did not overlap, we could not determine whether they correspond to a single gene. RT-PCR analysis confirmed that the hydrophobin-3 gene was repressed in Cd-treated mycelia and the downregulation of hydrophobin was even more pronounced after 72 h of treatment with 5 ppm Cd (Fig. 1).

Three clones encoding threonine dehydratase and showing between 3.0 and 3.4 reduced transcript levels were found, although we were not able to determine whether they correspond to a single gene. Transcripts encoding a cystathionine γ-synthase were downregulated 1.5-fold. With regard to the sulfur metabolism, γ-glutamylcystein, GSH and an unknown Cys-containing compound, unrelated to phytochelatins, increased by 2.3-, 2.5- and 6.5-fold in comparison to Cd-less controls after a 24 h exposure to 5 ppm of cadmium added to the culture medium [24].

4. Discussion

We have previously demonstrated that *P. involutus* responds to metal by complex mechanisms involving aspecific binding onto cell walls [10] and induction of anti-oxidative stress enzymes [11]. In the present study, we show that induction of laccase activity and production of polyphenolic compounds are also an important determinant of the cellular response to metal in *P. involutus*. It has been previously shown that copper can induce laccase isozymes in *Pleurotus ostreatus* [25] and metal-responsive elements in *P. ostreatus* laccase gene promoters have been recently found [26]. This enzyme catalyzes the synthesis of melanins from phenolic substrates [27]. Fungal melanins possess many potential metal-binding sites, e.g., phenolic, carboxyl, amine and hydroxyl groups [2].

Further complexation of metals extracellularly by organic acids or within cells by complexing peptides such as MT may also occur in *P. involutus*. Organic acid excretion upon metal exposure has been demonstrated in plants [28] and in ectomycorrhizal fungi [29,30]. Taken together, these results indicate that organic acids are Cd-chelating agents and that production of organic acids by mycorrhizal fungi may be regarded as a detoxification mechanism towards cadmium. In the ectomycorrhizal fungus *Rhizopogon roseolus*, the excretion of organic acids was coupled to proton release in the rhizosphere [31]. Interestingly, transcripts encoding an ATPase excreting H^+ were also upregulated by 2.1-fold in Cd-treated mycelia of *P. involutus*. Although the functions of α-galactosidase, fructose-bisphosphate aldolase, aconitase, and H^+-ATPase are not intimately related within the cellular machinery, the results presented here suggest that activation of carbon metabolizing enzymes may be needed to ensure organic acid production by *P. involutus*. Furthermore, downregulation of enzymes involved in the utilization of Asp (threonine dehydratase and cystathionine γ-synthase) may efficiently redirect carbon skeletons from Asp to the TCA cycle. Threonine dehydratase utilizes threonine to form isoleucine, threonine deriving from *O*-phosphohomoserine. Cystathionine γ-synthase combines *O*-phosphohomoserine with Cys to form cystathionine. *O*-phosphohomoserine derives from Asp and, therefore, these two enzymes belong to the biosynthetic pathway of the so-called Asp family of amino acids.

Metallothioneins are small, Cys-rich polypeptides that can bind essential metals, e.g., Cu and Zn, as well as non-essential metals such as Cd. A Cd-resistant strain of *S. cerevisiae* synthesized a cytoplasmic Cd-binding protein, which exhibits the characteristics of a metallothionein when grown in Cd-containing medium [2]. Using an improved HPLC method for the simultaneous measurement of thiol-containing compounds from cysteine and its derivatives (γ-glutamylcysteine, glutathione) to higher molecular mass (phytochelatins, metallothioneins), we found that glutathione and γ-glutamylcysteine contents increased when the ectomycorrhizal fungus *P. involutus* was exposed to cadmium [24]. An additional compound, with a high molecular mass, most probably related to a metallothionein, increased drastically in mycelia exposed to cadmium [24]. It is interesting to note that the induction of Cys synthase expression was not observed in our study, although it has been shown to be a key enzyme to supply free Cys for the enhanced synthesis of Cd^{2+} sequestering molecules such as GSH and phytochelatins in plants and some yeasts. Instead, the present study suggest that, as an alternative mechanism for the chelation of Cd^{2+} with Cys-rich compounds, the activity of other Cys-utilizing enzymes (cystathionine synthase) or the synthesis of Cys-riched proteins (hydrophobins) may be efficiently reduced, thus redirecting Cys to the manufacture of these Cys-enriched compounds. We found a downregulation of genes coding hydrophobins, which are a family of small hydrophobic Cys-rich proteins implicated in various developmental processes such as the emergence of aerial hyphae [33]. Indeed, growth of mycelial cultures of *P. involutus* in the presence of cadmium leads to an easily wettable phenotype (not shown). More specifically, the abundance of the *Schizophyllum commune* hydrophobin 3 mRNAs is strongly correlated with development of the mycelia, low abundance of Sc3 mRNA being correlated with the absence of aerial hyphae [34].

Related to the control of metal-responding genes, we found that AP-1 was upregulated in *P. involutus*. The *S. cerevisiae* yAP-1 is a transcriptional regulatory protein involved in cadmium resistance, which controls a number of metal response genes such as thioredoxin, YCF1 and GSH1 [23,32]. In this regard, it is noteworthy that a thioredoxin was also upregulated in our study, although not presented in Table 1 because of a low *P* value associated with the *t* test.

This work provides new insights into the strategy adopted by ectomycorrhizal fungi to deal with toxic metals, thus improving both our understanding of the ecology and the agricultural applications of these useful fungal symbionts. Further studies are now needed to delineate the functions of both known and novel genes that are differentially expressed during metal exposure.

Acknowledgements: Financial supports from the Région Lorraine and from the Agence de l'Environnement et de la Maitrise de l'Energie (ADEME) are gratefully acknowledged. Many thanks to Patrice Godard, Mélanie Morel, Sébastien Duplessis and Annegret Köhler for

helpful discussions and to Christine Delaruelle for technical assistance in sequencing using the DNA sequencing facilities at INRA-Nancy (IFR 110).

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